

## A METHOD FOR THE PREPARATION OF CRYSTALLINE OXYHEMOGLOBIN.

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Available methods for the preparation of crystalline oxyhemoglobin may be divided into two groups, in the first of which alcohol or ether, or both, are used as aids to crystallization, and in the second of which the use of these solvents is avoided. Possible objections to the use of alcohol were early pointed out by Hüfner (1), and Mayet (2) emphasized the advantages of "benzine" over ether. Although these workers, as well as Bohr (3) and Torup (4), made preparations in which the use of either or both of these substances was avoided, the first systematic attempt to work out a method without their aid seems to have been the recent one of Dudley and Evans (5). Their procedure involves pressure dialysis of the washed red cells of horse blood, crystallization of the oxyhemoglobin in the dialysate by oxidation, and recrystallization of the product from water by reduction *in vacuo* at 37° and subsequent oxidation. Data as to the yield and purity of the product are not given.

In the hope of obtaining fairly large amounts of oxyhemoglobin in the highest possible state of purity, the Dudley and Evans method was first employed, using a modification suggested by Adolph and Ferry (6); namely, final dialysis against water saturated with carbon dioxide. The oxyhemoglobin obtained in this way crystallized as large plates, instead of the needles reported by Dudley and Evans. Furthermore, the insolubility of the plates and their consequent resistance to reduction not only justified the belief that isoelectric oxyhemoglobin was being dealt with, and that Dudley and Evans were probably working with a more soluble salt, but rendered the use of the method impossible for the purpose in view, and it was abandoned in favor of a different principle.

The method now proposed depends upon observations that suspensions of washed dog or horse red cells crystallize rapidly and almost completely in the presence of toluene when saturated with carbon dioxide and oxygen, and that the resulting oxyhemoglobin may be recrystallized by solution with the aid of sodium carbonate and reprecipitated with carbon dioxide.

The use of toluene was found to hasten markedly the crystallization of the oxyhemoglobin of the corpuscles owing to its disintegrating effect on the cells themselves. While its hemolytic action is slower than that of ether, its use obviates the chief disadvantages of the latter; namely, solubility in water, and the presence of peroxides and other reactive substances which may alter oxyhemoglobin. The carbon dioxide shifts the reaction in the acid direction past the isoelectric point of oxyhemoglobin, so that the crystals obtained are oxyhemoglobin uncombined with alkali. By thus promoting the crystallization of the oxyhemoglobin the acidification also aids in the original disintegration of the corpuscles. Saturation with pure carbon dioxide would, however, drive oxygen out of the solution and change the oxyhemoglobin to the reduced form, which is too soluble to crystallize readily. In order to obviate this difficulty 1 part of oxygen was mixed in a cylinder with 4 parts of carbon dioxide for the saturation. Such a mixture may be passed through oxyhemoglobin solutions indefinitely without reduction.

Removal of the salts is accomplished by the simplified form of pressure dialysis<sup>1</sup> suggested by Adair, Barcroft, and Bock (7), *after* the desired number of recrystallizations has been carried out. Two recrystallizations have been deemed sufficient in this laboratory, but for many purposes the oxyhemoglobin will undoubtedly be found pure enough after the first recrystallization. On the other hand, the losses involved in each recrystallization, while appreciable, are not sufficiently large to preclude three or even four recrystallizations.

Three precautions have been found essential: (a) All operations are carried out in the cold, centrifugation being a possible exception if a centrifuge in a cold room is not available. (b) The oxyhemoglobin is not allowed to become dry, owing to the resultant change, noted by Bohr (8), into a modification in which the

<sup>1</sup> Except that the membranes are not sterilized.

TABLE I.

Preparation No.	Volume of blood.		Total oxyhemoglobin content.	Preliminary separation in centrifuge.	First recrystallization.			Second recrystallization.			Yield.		Oxygen capacity.	Conductivity at 25° of saturated aqueous solution.*	Concentration of saturated aqueous solution.
	cc.	gm.			Volume of H <sub>2</sub> O.	Total volume of suspension.	N Na <sub>2</sub> CO <sub>3</sub>	Volume of H <sub>2</sub> O.	Total volume.	N Na <sub>2</sub> CO <sub>3</sub>					
Dog 8.	150	29.4	+	50	85	10-11	30	65			7.8	26.5	96.3	$6.6 \times 10^{-5}$	
" 9.	295	55.5	-	50	200	6-6.5	30	80	8.5-9		13.1	23.6	100.0	—	
" 10.	300	45.9	-	70	160	10-11	30	90	9-10		14.4	31.4	96.3	$3.9 \times 10^{-5}$	4.3
" 11.	465	76.3	+	100	230	18-22.5	70	170	18-19		29.2	38.3	99.3	$5.4 \times 10^{-5}$	
" 12.	248	37.3	-	60	140	10-11	35	80	7		8.95	24.0	98.0	$5.2 \times 10^{-5}$	3.9
Horse 6.	605	72.0	-	40	210	15	35	100	5-6		18.75	26.0	97.7	$6.6 \times 10^{-5}$	2.7

\* For the preparation of the solutions for conductivity determinations, see p. 38.

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oxygen is not reactive. (c) During the various manipulations on the acid side of the isoelectric point, before the final dialysis, care is taken to have an excess of carbon dioxide constantly present. If the carbon dioxide tension is permitted to fall, part of the oxyhemoglobin is redissolved as alkali salt.

The purity of the oxyhemoglobin obtained by the present method has been controlled by a determination of the ratio of the oxyhemoglobin present, as determined by Van Slyke and Stadie's procedure (9), to the total hemoglobin pigments present, determined as cyanhemoglobin by Stadie's method (10). As will be seen in Table I, preparations of 96 to 100 per cent of the theoretical oxygen capacity were obtained. The relative freedom of the product from salts was controlled by conductivity measurements of saturated aqueous solutions, the values obtained being also given in the table.

#### EXPERIMENTAL.

Oxalated or defibrinated dog or horse blood of known oxyhemoglobin content is centrifuged and the plasma or serum and the layer of white cells are removed. The red cells are then washed three times with chilled 0.85 per cent sodium chloride solution, after which the supernatant liquid usually gives at most only a faint haze when a test portion is boiled. The cells are then rinsed into a flask with a few cubic centimeters of water. The vessel is cooled in ice water, and a steady stream of a mixture of 4 parts of carbon dioxide to 1 part of oxygen passed in. Toluene is, meanwhile, added in amount equal to about one-seventh of the volume of corpuscles, and the mixture is stirred with the gas inlet tube until it becomes pasty. Passage of the gas is continued for a few minutes, with vigorous stirring, after which the flask is stoppered tightly with a rubber stopper and allowed to stand over night in the ice box. This is often long enough to complete the process of disintegration of the cells and crystallization of the oxyhemoglobin, but if many intact cells are still to be seen under the microscope the treatment with carbon dioxide and oxygen is repeated and the flask allowed to stand a day or two longer.

The consistency of the resulting mixture depends somewhat upon the extent to which the red cells have been packed in the centrifuge and upon other factors which have not been determined.

If the mixture is sufficiently thin it may be centrifuged with advantage in chilled tubes in a cold room, separating into an upper layer of toluene and cell fragments, an intermediate layer of clear solution, and a lower layer of oxyhemoglobin crystals. The two upper layers are poured off and the crystals drained in the ice box on a chilled porous plate, the surface layer being renewed constantly as it dries out, in order to avoid possible conversion of the oxyhemoglobin into a form in which the oxygen is less reactive. During this process a slow stream of carbon dioxide should be directed over the surface of the plate, otherwise a portion of the oxyhemoglobin will redissolve as carbon dioxide evaporates from the mixture. When drainage is as complete as possible, the oxyhemoglobin is scraped into a chilled mortar and ground to a smooth paste with sufficient ice-cold water to bring the final volume up to three to three and a half times (in cubic centimeters) the weight in grams of oxyhemoglobin present in the original blood.

In case the crude mixture of crystals, toluene, and cell fragments is too thick to permit centrifugation, the entire mass is transferred to a porous plate, using the same precautions as given above. Under these conditions the process of drainage takes much longer and cannot be carried to completion owing to the emulsion formed by the toluene. On the other hand, the product, being less compact, is easier to grind to a smooth paste with water, and the toluene and cell fragments may be removed during the first recrystallization. The final volume in this case should be kept as close as possible to that given above.<sup>2</sup>

The thin paste of crude oxyhemoglobin is transferred to a beaker, set in ice water, and titrated to minimum turbidity with normal sodium carbonate solution. During the addition of carbonate the mixture is stirred thoroughly, and any lumps which may remain are disintegrated. The amount of sodium carbonate necessary is greatest, of course, when the crude crystals have been thoroughly drained and contain as little as possible of the bicarbonate and

<sup>2</sup> An alternative method, which is quite satisfactory in the case of dog blood, but is very slow in the case of horse blood, is to filter the entire mass in the ice box through silk, using as large a Buchner funnel as possible, and observing the precautions given below for filtering oxyhemoglobin suspensions.

salts of the mother liquor. In this case the final concentration of alkali added as carbonate is approximately 0.1 N. If the toluene and cell fragments have been separated previously by centrifugation and if enough water is present, a fairly clear, deep red solution will result, but if too little water is used a crystalline precipitate of what appears to be sodium oxyhemoglobinate will remain. In this case, and also in the case in which the toluene and cell fragments are still present, the carbonate solution is added to the point of minimum turbidity, after which 1 or 2 cc. more are added in order to make sure of an excess.

The solution is next centrifuged, and any toluene and cell fragments on top are sucked off through a capillary tube, a process which can generally be accomplished without appreciable loss of the actual oxyhemoglobin solution. If loss should occur, however, the mixture which has been sucked off may be whirled again and the clear oxyhemoglobin solution added to the main portion. If enough alkali has been added and there is still a crystalline deposit in the centrifuge tubes, too little water is present, and the precipitate may be dissolved in the minimum amount of water and the solution added to the main portion. This precipitate, which is usually encountered at this point only when dog blood has been used, appears to be sodium oxyhemoglobinate, for it is readily soluble in water with a bright red color, it has a characteristic crystalline form, and, finally, yields crystals characteristic of dog oxyhemoglobin when a concentrated aqueous solution is saturated with carbon dioxide-oxygen mixture and allowed to stand in the cold. Further investigation of this salt will be undertaken.

The oxyhemoglobin solution is next chilled and a stream of the carbon dioxide-oxygen mixture passed in until crystallization *begins*, after which the flask is tightly stoppered and set in the ice box. Often within a few minutes the oxyhemoglobin has set to a solid cake of long, flat, scarlet needles in the case of dog oxyhemoglobin, and dark red, glistening, broader plates, often diamond-shaped or hexagonal, in the case of horse oxyhemoglobin.

After standing over night in the ice box the crystals are sucked off on hardened paper in a Buchner funnel (the 5 inch size is adequate for the oxyhemoglobin from 300 cc. of blood). The filtration is carried out in the ice box, with a slow stream of

carbon dioxide passing into the funnel. The surface is kept moist by renewal with a spatula as it dries out, and when this is no longer possible, a few cc. of water saturated with carbon dioxide are sucked through with the same precautions, after which the filtration is stopped. The entire process usually takes less than 1 hour.

For many purposes the oxyhemoglobin is undoubtedly sufficiently pure at this point, and in one experiment which was interrupted at this stage the amount of crystalline oxyhemoglobin recovered was 46 per cent of the amount present in the original blood, as determined by the oxygen capacity.

For further purification the recrystallization process is repeated. The crystalline cake is transferred to a chilled mortar and again ground to a smooth paste with cold water. The volume of the suspension thus obtained should be about 0.7 of that employed for the first recrystallization if the toluene and cell fragments have been initially removed by centrifugation, and from 0.4 to 0.6 as large if the removal of the upper layer was accomplished during the first recrystallization. The larger fraction will, of course, be necessary when drainage of the original crystallized cell mixture on the porous plate has been most complete. The suspension of oxyhemoglobin is dissolved with normal sodium carbonate solution, centrifuged, reprecipitated with the carbon dioxide-oxygen mixture, and collected, at every step with the same precautions as in the first recrystallization.

If salt-free oxyhemoglobin is desired, the crystals are ground with the minimum amount of cold water to a paste which will just flow easily, saturated in the cold with the carbon dioxide-oxygen mixture, transferred at once to narrow collodion dialysis bags, and dialyzed under pressure in the ice box against water saturated with carbon dioxide-oxygen mixture. Dialysis for 3 or 4 days, the carbon dioxide-oxygen-saturated water and the positions of the bags being changed daily, is sufficient to bring the conductivity down to the values given in Table I. The dialysis tubes found most suitable in this laboratory were made in 50 cc. test-tubes with one of the eminently satisfactory collodion mixtures proposed by Eggerth (11), namely, a solution of 7 gm. of "Parlodion" in 60 cc. of ether, 30 cc. of alcohol, and 10 cc. of glacial acetic acid. Dialysis under pressure was accomplished by simply

closing the ends of the tubes with tightly screwed, rubber-faced screw pinch-cocks, as proposed by Adair, Barcroft, and Bock (7).

At the end of the dialysis the contents of the bags, which still retain their crystalline structure, are sucked off in the ice box on hardened paper in a Buchner funnel, using, as before, the precaution of keeping the surface layer moist. The use of carbon dioxide at this stage is unnecessary, as the oxyhemoglobin remains sparingly soluble in the absence of alkali and salts.

The conductivity values were obtained by grinding the product in a chilled mortar with ice water, centrifuging the resulting suspension, pouring off at temperatures ranging from 19 to 28°, and measuring the conductivity of the clear supernatant solution after evacuating a few times to remove any carbon dioxide present. The oxyhemoglobin content of the resulting solutions is given in Table I.

The purified oxyhemoglobin was dissolved either with the aid of sodium carbonate solution or with a sufficient excess of N/7 sodium hydroxide to bring the final concentration of alkali to 0.03 or 0.04 N, and the solution was filtered through a small, loose plug of washed cotton into a volumetric flask of appropriate size and made up to the mark. The yield of oxyhemoglobin was calculated from the oxygen capacity of the resulting solution, and varied between 23 and 38 per cent of the total originally present in the blood used. The purity of the product was determined by comparing the oxygen capacity with the total hemoglobin content as determined by Stadie's (10) methemoglobin method,<sup>3</sup> and the ratio of oxyhemoglobin to total hemoglobin pigments was found to vary between 96 and 100 per cent.<sup>4</sup>

The entire process of preparation of the dialyzed oxyhemoglobin can scarcely be completed in less than a week. On the other hand, it is not desirable to let preparations stand unduly long at the various stages of purification, for if the process is extended

<sup>3</sup> It is advisable to check up the cyanhemoglobin standard at least every 2 weeks, as the color tends to deepen, even in the ice box. The color of the chilled standard also changes with rise in temperature, so that the solution should be allowed to come to room temperature before comparisons are made.

<sup>4</sup> The experiments recorded in this paper were all performed before the hot weather set in. Since that time yields and oxygen capacities have occasionally dropped as much as 5 per cent below the values given above.



for much over 2 weeks there is a noticeable diminution of the oxygen-binding power of the product. Also, when solutions saturated with the carbon dioxide-oxygen mixture are allowed to stand it is desirable to resaturate with the gas mixture at least every other day in order to compensate for leakage.

The stability to be expected of the solutions of oxyhemoglobin obtained by this method is indicated in Fig. 1, in which a steady diminution of the oxyhemoglobin content is shown amounting roughly to 1 per cent per day. The total hemoglobin content

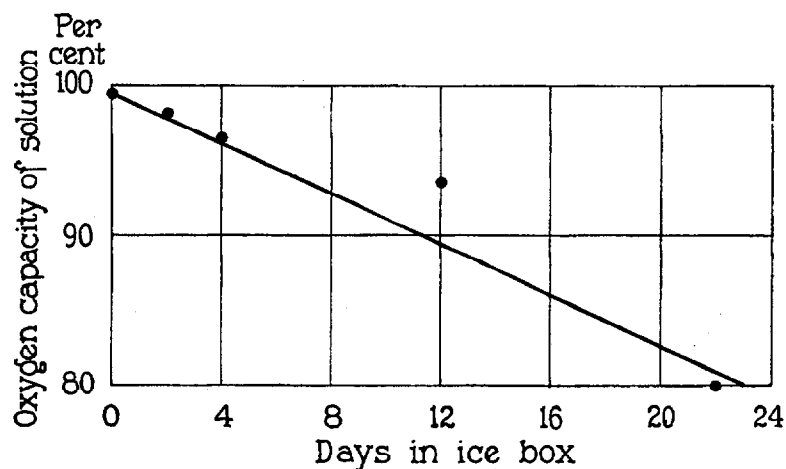


FIG. 1.

of the original solution was 13.8 gm. per 100 cc. and the oxyhemoglobin content 13.7 gm., while the latter had fallen to 11.05 gm. at the end of 22 days.

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